

A microfabricated platform for the study of the influence of substrate stiffness on  
topographically-guided cancer cell migration

Undergraduate Engineering Honors Research Thesis

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## Abstract

Cell migration is an essential aspect of embryogenesis, immune responses, and wound healing, but it is also a defining characteristic of highly malignant and aggressive forms of cancer, such as breast adenocarcinoma. Breast cancer cells prefer to migrate along highly oriented collagen fibers within breast tissue. However, the migration pattern of breast cancer cells could also be impacted by the stiffness of the surrounding mammary tissue. To better characterize the migration of different types of cancer, we developed an *in vitro* system to study the migration of cancer cells along micropatterns on substrates of varying stiffness. Previous work investigated the migration of glioma, lung, and colon cancer cells on polymers including polystyrene (PS) and polydimethylsiloxane (PDMS, silicone rubber), but these substrates are orders of magnitude stiffer than native tissues. To address this shortcoming, polyacrylamide (PA) gels were created with Young's moduli of 1 kPa, 10 kPa, and 120 kPa. Existing PA recipes were adjusted so that 5  $\mu\text{m}$  lines were maintained in the PA gels using a patterned SU8 wafer as a mold. Preliminary studies were conducted using metastatic, migratory breast cancer (MDA-MB-231) cells. It was found that the cells migrated at a velocity of  $0.65 \pm 0.13 \mu\text{m}/\text{min}$  ( $39 \pm 7.8 \mu\text{m}/\text{hr}$ ) on the 10 kPa polyacrylamide gels. It was not possible to obtain migration velocity data for MDA-MB-231 cells on the 1 kPa and 120 kPa gels due to pattern swelling (1 kPa) and poor cell attachment (120 kPa). Future studies using glioblastoma cells lines, patient-derived breast cancer cells, and patient-derived glioma cells could ultimately lead to improved treatment options and prognoses for patients.

## Introduction

Cellular migration is integral for many processes such as embryogenesis, immune response, and wound healing.<sup>1</sup> However, cell migration is also partly responsible for the spread and metastasis of certain forms of cancer. Cell migration occurs through four processes: (1) protrusion of the leading edge of the cell, (2) adhesion to the surrounding extracellular matrix (ECM), (3) movement of the cell body, and (4) retraction of the trailing edge of the cell.<sup>2</sup> Cells migrate by forming and breaking attachments to the ECM. However, in order to move, the cells must create spatial asymmetry by reorganizing the actin cytoskeleton.<sup>3</sup> Following attachment to a substrate, F-actin polymerizes and pushes the leading edge of the cell membrane forward forming lamellipodia. The lamellipodia allow the cells to form new attachments at their front ends. After the new attachments are formed, attachments at the rear of the cell are released, and the cell moves forward.<sup>3</sup> Throughout the migration process, cells interact with the ECM through integrin receptors. FAK and other intracellular proteins allow ECM interactions to be coupled with signaling events. Integrin-ECM interactions and the signaling cascades they trigger are partly responsible for cellular migration pattern and speed.<sup>3</sup>

Cancer cell migration patterns can be classified into two categories: single-cell migration and collective migration.<sup>4</sup> Single-cell migration occurs when single cells break off of the bulk tumor and invade the surrounding tissue by using an amoeboid strategy (integrin-independent) or a mesenchymal strategy (integrin-dependent).<sup>4</sup> The amoeboid strategy is common in lymphomas while the mesenchymal strategy is common in glioblastoma multiforme and fibrosarcomas.<sup>4</sup> In collective migration, cell-cell adhesions in cell groups lead to actin filament polymerization along cell junctions, which results in a multicellular contractile body that branches out from the tumor in sheets and strands.<sup>4</sup> Collective migration is common in invasive epithelial cancers such

as mammary carcinoma.<sup>4</sup> Cancer cells may exhibit both types of migration patterns or transition between pattern types. One such transition is termed the epithelial-mesenchymal transition (EMT) in which epithelial cancer cells transition from collective migration patterns to single-cell migration patterns.<sup>4</sup> This transition may occur in breast, colon, lung, or prostate cancer and often marks an increase in metastasis leading to a poor prognosis.<sup>4</sup>

Since cancer cell migration influences dispersion and metastasis, it is important to fully understand the factors that influence cell migration speeds and behaviors. Previous studies have shown that cells may actively probe and respond to mechanical cues in their surrounding environment.<sup>5</sup> Furthermore, the forming and breaking of attachments as well as the reorganization of the actin cytoskeleton during cell migration may be influenced in part by the stiffness of the surrounding ECM and tissue. Several studies have found that U87 and U373 glioblastoma cell migration speed increases with substrate elastic modulus on substrates of 0.08-119 kPa.<sup>6</sup> These findings suggest that migration speed is influenced by substrate stiffness and may be biphasic. Bangasser, *et al.* developed a computational motor-clutch model to analyze the relationship between cell migration and substrate stiffness. In this model, cell adhesion molecules are modeled as clutches and myosins are modeled as motors.<sup>6</sup> The motor-clutch model suggests that there is an optimal substrate stiffness for cell migration. This optimal stiffness occurs when clutches (cell adhesion molecules) are used to the fullest extent and motors (myosin) are resisted to the fullest extent.<sup>6</sup> Bangasser suggests that by decreasing the number of cell adhesion molecules and myosin motors, tumor cell migration could be slowed, since the optimum stiffness would be shifted away from the stiffness of the microenvironment.<sup>6</sup> Shifting the optimum migratory stiffness for tumor cells could present a new target for cancer therapies.

The motor-clutch computational model of Bangasser, *et al.* was based on experimental data from *in vitro* migration studies. Several substrates that mimic the cellular microenvironment have been developed to study cancer cell migration *in vitro*. Pathak and Kumar developed a system to study the effects of matrix stiffness and confinement on glioblastoma multiforme cell migration. Microchannels with varied width between 10 and 40  $\mu\text{m}$  were fabricated from polyacrylamide with stiffness of 0.4, 10, and 120 kPa.<sup>7</sup> The migration rate of the confined cells was found to increase as substrate stiffness increased. However, this *in vitro* system did not contain microscale features that mimicked the scale of structures in native brain tissue. Gallego-Perez, *et al.* developed an *in vitro* system consisting of a polystyrene substrate with 5 x 5  $\mu\text{m}$  ridges spaced by 5 or 45  $\mu\text{m}$  grooves. The micropatterned polystyrene was used to study the migration velocity and patterns of glioma, lung, and colon cancer cells.<sup>8</sup> However, the stiffness of the polystyrene (~3 GPa) was orders of magnitude higher than that of native tissues.

The system described in this thesis aims to incorporate the tunable stiffness of the Pathak and Kumar substrate with the micropattern of the Gallego-Perez, *et al.* substrates with the goal of mimicking migration conditions of cancer cells *in vivo*. Polyacrylamide gels were patterned with 5  $\mu\text{m}$  lines with 5  $\mu\text{m}$  spacing as this scale has been previously used to probe cancer cell motility with single-clone resolution.<sup>8</sup> The micropatterned lines recapitulate structures in native tissue along which cancer cells preferentially migrate. For example, glioblastoma cells preferentially migrate along white matter tracts and blood vessels while breast cancer cells migrate along collagen fibers.<sup>1,3</sup> The stiffness of the gels was tuned to 1, 10, and 120 kPa as these values fall within a range reported for a variety of native tissue types.<sup>9</sup> The gels will be used to determine the optimal migration stiffness for breast cancer cells and glioblastoma multiforme cells. Once the optimal stiffness is known, the myosin and actin within the cells can be targeted to shift the



optimal migration stiffness and slow the migration speed of the cancer cells. By gaining a better understanding of the factors that influence cancer cell migration, treatments can be developed that lead to better prognoses for patients.

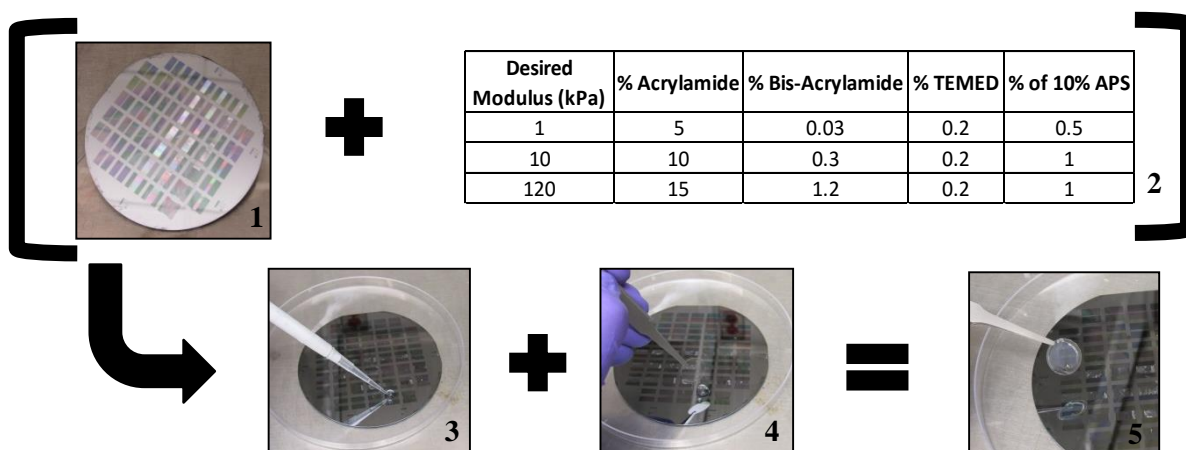
The current project was centered on two aims: (1) create polyacrylamide gels with elastic moduli of 1, 10, and 120 kPa with a pattern of 5  $\mu\text{m}$  lines; and (2) use MDA-MB-231 cells as a model of highly aggressive tumor cells for migration studies. Polyacrylamide gels of all three stiffness values were fabricated successfully, and 10 kPa gels were used for migration studies with the MDA-MB-231 cells. The results of this study will allow for an enhanced understanding of the factors influencing breast cancer cell migration *in vivo* and provide a basis to apply the platform to study other models of highly invasive cancerous cells, such as gliomas.

## Methodology

### *Polyacrylamide Gel Preparation*

Micropatterned lines (5  $\mu\text{m}$  lines with 5  $\mu\text{m}$  spacing) of polyacrylamide were fabricated by curing polyacrylamide on a SU8 silicon mold that was created using standard UV photolithography. 40% acrylamide solution (Santa Cruz Biotechnology Inc.) was mixed with 2% bis-acrylamide solution (Research Products International Corp.) at different ratios to obtain the desired Young's modulus. Stock solutions of 5% acrylamide with 0.03% bis-acrylamide, 10% acrylamide with 0.3% bis-acrylamide, and 15% acrylamide with 1.2% bis-acrylamide, all in distilled water, were used to produce gels with Young's moduli of 1, 10, and 120 kPa, respectively. To polymerize the gels, 10% ammonium persulfate (APS) (Sigma-Aldrich) in distilled water was added to the stock solutions at concentrations of 0.5% for the 1 kPa gels and 1.0% for the 10 kPa and 120 kPa gels. Undiluted tetramethylethylenediamine (TEMED) (Research Products International Corp.) was also added to the stock solutions at a concentration of 0.2% for the 1, 10, and 120 kPa gels.

To form the gels, 100  $\mu\text{L}$  of an acrylamide/bis-acrylamide stock solution containing the APS and TEMED initiators, was pipetted onto the SU8 silicon mold. A coverslip (15 mm diameter) that was previously washed in 70% ethanol and 0.1N NaOH and then incubated with 3-(2-aminoethylamino)propyltrimethoxysilane (Fluka) followed by 0.5% glutaraldehyde (Fisher Scientific) in PBS was then placed immediately over the polymerizing solution. After polymerization (30-45 minutes), the edges of the gel retracted from the coverslip, and the gel was removed from the SU8 wafer using forceps. The polyacrylamide gels remained attached to the functionalized coverslips and were stored in distilled water at 4  $^{\circ}\text{C}$ . A more detailed description of the polyacrylamide gel fabrication protocol can be found in Appendix A.



**Figure 1:** A schematic of the polyacrylamide gel fabrication process. The table lists the proportions of acrylamide and bis-acrylamide needed to create an aqueous stock solution for each stiffness value. The reagent concentrations listed should be added to the stock solution to promote polymerization of the gel. (1) SU8 silicon master, (2) table of reagent concentrations, (3) pipetting the stock solution onto the silicon master, (3) placing a treated coverslip, (4) removing the cured polyacrylamide gel.

### Cell Culture

The gel substrates were placed in a 12-well plate and washed with 70% ethanol and PBS. The gels were then incubated with fetal bovine serum (FBS) (Sigma) for 24 hours before MDA-MB-231 cells (Sigma-Aldrich) were seeded on the surface at a density of 14,000 cells/cm<sup>2</sup>. The cells were incubated at 37 °C and 5% CO<sub>2</sub> for an additional 24 hours before performing a migration assay. MDA-MB-231 cells were maintained in Eagle's minimum essential medium (EMEM) (Sigma), supplemented with 10% FBS and 1% penicillin streptomycin (BioWhittaker) at 37 °C and 5% CO<sub>2</sub>.

### Migration Assay

The cells were imaged in CO<sub>2</sub>-independent media (Gibco) for 10 hours using a phase contrast microscope (Eclipse Ti-E, Nikon) fitted with a culture chamber at 37 °C (Okolab). Images were taken every 10 minutes and then post-processed using the manual tracker plug-in in

Fiji (<http://fiji.sc/>). A more detailed description of the post-processing protocol can be found in Appendix A. Observations of MDA-MB-231 cells on tissue culture polystyrene were used as a control.

## Results

### *Polyacrylamide Gel Preparation*

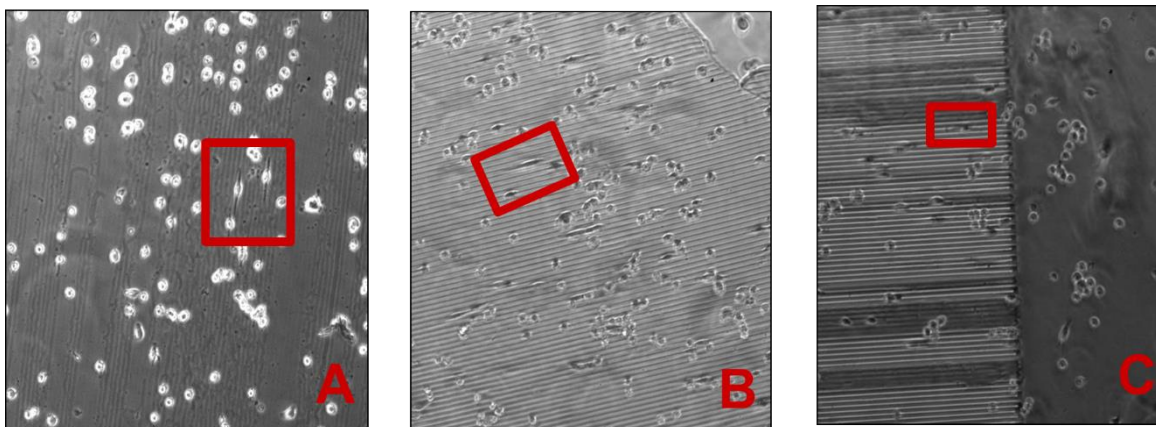
The 1 kPa, 10 kPa, and 120 kPa polyacrylamide gels retained the 5  $\mu\text{m}$  line pattern following polymerization. As shown in Figure 2, the quality of the pattern varied depending on the stiffness of the gel. The micropatterned lines on the 10 kPa and 120 kPa gels were crisp and distinct, while the micropatterned lines on the 1 kPa gel were wavy and faint. 10 kPa gels were chosen for the migration assay due to ease of fabrication and pattern robustness following the polymerization of the polyacrylamide.



**Figure 2:** Images of 5  $\mu\text{m}$  lines on (A) 1 kPa, (B) 10 kPa, and (C) 120 kPa polyacrylamide (PA) gels at 20x magnification.

### *Cell Culture*

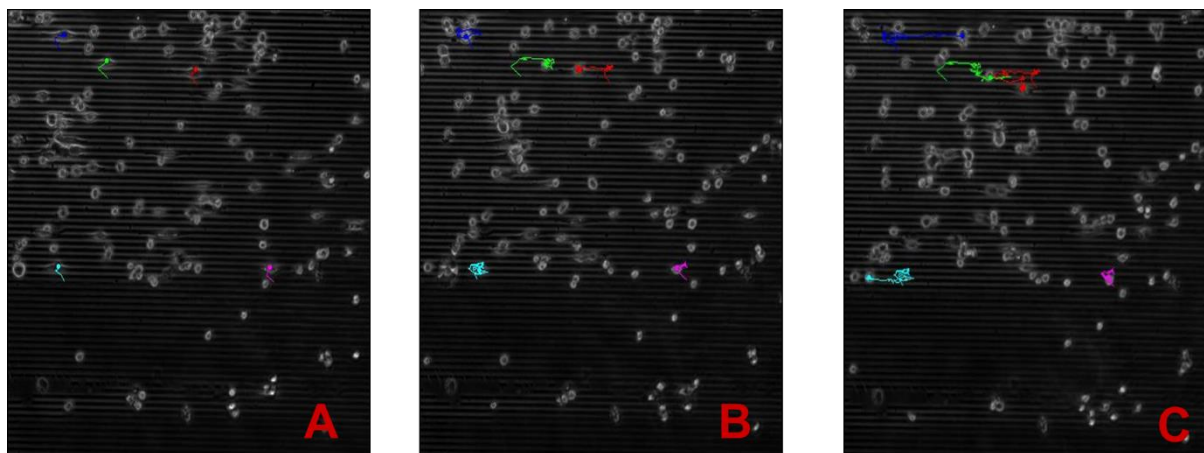
As shown in Figure 3, the morphology of the MDA-MB-231 cells varied depending on the polyacrylamide gel substrate. On the 1 kPa gels, individual cells elongated the most so that lamellipodia were distinctly visible. On the 10 kPa gels, individual cells elongated, but distinct lamellipodia were not visible. Cells on the 120 kPa gels elongated but did not become as flattened as the cells on the 10 kPa gels. Counts of elongated cells on the lines were not collected, but qualitatively the greatest number of cells elongated on the 10 kPa gel, followed by the 120 kPa gel and the 1 kPa gel.



**Figure 3:** Images of 5  $\mu\text{m}$  lines on (A) 1 kPa, (B) 10 kPa, and (C) 120 kPa polyacrylamide (PA) gels with MDA-MB-231 cells seeded on the surface (10x magnification). The red boxes highlight cells that have elongated along the length of a micropatterned line.

### *Migration Assay*

The velocity of ten cells on a single 10 kPa polyacrylamide gel was calculated, and the migration pattern of each of these cells was traced. Figure 4 shows the migration pattern of five of these cells after 2, 5, and 10 hours into the migration assay. The cell traced in blue in Figure 4 moved along a single micropatterned line throughout the entire ten hours, but the other cells migrated across several of the 5  $\mu\text{m}$  lines. The velocities of these ten cells were then averaged to achieve an overall velocity of  $0.65 \pm 0.13 \mu\text{m}/\text{min}$ . The velocities of each individual cell can be found in Table 1.



**Figure 4:** Images of MDA-MB-231 cell migration with traces on a 10 kPa gel after (A) 2 hours, (B) 5 hours, and (C) 10 hours (10x magnification). The traces were added using the manual tracker plug-in in Fiji.

**Table 1:** Average velocities for MDA-MB-231 cell migration on a 10 kPa polyacrylamide gel.

| Cell  | Average Velocity ( $\mu\text{m}/\text{min}$ ) |
|---|---|
| 1   | 0.66  |
| 2   | 0.80  |
| 3   | 0.71  |
| 4   | 0.76  |
| 5   | 0.42  |
| 6   | 0.74  |
| 7   | 0.69  |
| 8   | 0.66  |
| 9   | 0.39  |
| 10  | 0.69  |
| <b>Overall Average Velocity (<math>\mu\text{m}/\text{min}</math>)</b> |   |
| $0.65 \pm 0.13$   |   |

## Discussion

### *Polyacrylamide Gel Preparation*

Existing polyacrylamide gel recipes in the literature had to be modified so that the gel retained the 5  $\mu\text{m}$  pattern following polymerization. The concentrations of acrylamide and bis-acrylamide were not adjusted, but the initiator concentrations were increased. The concentration of TEMED used to initiate polymerization was quadrupled for the 1 kPa, 10 kPa, and 120 kPa gels, and the concentration of ammonium persulfate was doubled for the 10 kPa and 120 kPa gels.<sup>10</sup> The concentration of ammonium persulfate suggested by Higuera-Castro, *et al.* was used to initiate polymerization of the 1 kPa gels.<sup>10</sup> Due to this change in initiator concentrations, the stiffness of the gels may vary slightly from the literature values of 1, 10, and 120 kPa. An increased concentration of TEMED and ammonium persulfate results in a decreased average polymer chain length after polymerization.<sup>11</sup> The relatively short polymer chains lead to an increase in gel turbidity and a decrease in gel elasticity.<sup>11</sup> Since the initiator concentrations were increased to create the micropatterned gels, the actual stiffness of the gels may be greater than 1, 10, and 120 kPa. Atomic force microscopy (AFM) will need to be used to determine the actual elastic modulus of the gels. However, despite changes to literature recipes, the gels should differ from each other in stiffness by at least one order of magnitude.

PDMS stamps with the 5  $\mu\text{m}$  lines were originally used to micropattern the polyacrylamide gels. However, the PDMS hampered the acrylamide polymerization reaction, so the gels did not cure. The exact mechanism by which PDMS poisons the acrylamide reaction is unknown; however, it is most likely caused by diffuse oxygen in the PDMS. An etched silicon mold with 2  $\mu\text{m}$  lines was also used to pattern the polyacrylamide. The gels cured using this



mold, but pattern transfer did not occur. An SU8 silicon mold with 5  $\mu\text{m}$  lines was ultimately used to successfully pattern the polyacrylamide gels.

The completeness of the pattern transfer varied depending on the gel stiffness. The micropatterned lines on the 10 and 120 kPa gels were crisp and distinct, while the lines on the 1 kPa gel were wavy. The wavy lines on the 1 kPa gel could have been caused by swelling of the gel after it had polymerized. It was also difficult to de-mold the 1 kPa gels from the SU8 lines. Portions of the gel would stick to the mold, and would have to be released by immersing the gel in distilled water. The micropatterned features of the 1 kPa gel could have been damaged during this de-molding process.

### *Cell Culture*

At least two cells elongated on each type of polyacrylamide gel, but the majority of the cells remained circular. It was expected that the majority of the cells would elongate along the micropatterned lines, since this behavior was previously observed by Gallego-Perez, *et al.* The unexpected cell morphology could be explained by the surface functionalization: incubating the gels in FBS may not have been an ideal method to functionalize the surface of the gels. Breast cancer cells typically migrate along collagen fibers, so perhaps coating the surface of the polyacrylamide gels with collagen would promote cell elongation and migration.<sup>1</sup>

### *Migration Assay*

Migration velocities were obtained for MDA-MB-231 cells on 10 kPa gels, since these gels maintained the micropatterned features at the lowest possible stiffness value. The average cell velocity ( $0.65 \pm 0.13 \mu\text{m}/\text{min}$  or  $39 \pm 7.8 \mu\text{m}/\text{hr}$ ) fits with values previously observed for similar forms of cancer such as lung ( $41.8 \pm 4.6 \mu\text{m}/\text{hr}$ ), glioma ( $24.0 \pm 1.8 \mu\text{m}/\text{hr}$ ), and colon ( $26.7 \pm 2.8 \mu\text{m}/\text{hr}$ ).<sup>8</sup> However, the migration patterns of the MDA-MB-231 cells did not conform

to those reported for similar types of cancer.<sup>8</sup> Only one MDA-MB-231 cell migrated along a single 5  $\mu\text{m}$  line for the entire 10 hours of the migration assay, while directional persistence along a single micropatterned feature had been reported previously by Gallego-Perez, *et al.*<sup>8</sup> The cells may have moved between multiple lines, since the lines were only 5  $\mu\text{m}$  apart as opposed to up to 45  $\mu\text{m}$  apart in previous studies.<sup>8</sup> The migratory pattern exhibited by most of the cells in this study may actually be indicative of the migration pattern *in vivo*, since collagen fibers tend to be bundled. The 5  $\mu\text{m}$  spacing of the lines may more closely recapitulate this bundling of collagen fibers than the 45  $\mu\text{m}$  spacing used previously.<sup>8</sup>

A previous study by Wong, *et al.* showed that MDA-MB-231 cells utilize a mesenchymal migration pattern for single-cell migration.<sup>13</sup> Mesenchymal migration patterns were also observed in the migration assay described in this thesis; however, amoeboid migration patterns were also observed, since circular cells migrated along the micropatterned lines. Future studies could be conducted to determine the percentage of MDA-MB-231 cells that utilize a mesenchymal migration pattern and the percentage of cells that utilize an amoeboid pattern. Migration patterns could be assessed by examining actin polarization (present in mesenchymal migration) and integrin involvement, since mesenchymal migration is integrin-dependent and amoeboid migration is integrin-independent.

## Conclusions

1, 10, and 120 kPa polyacrylamide gels were successfully patterned with 5  $\mu\text{m}$  lines. MDA-MB-231 cells were then used in proof of concept migration studies to verify the micropatterned polyacrylamide substrates of varying stiffness. It was observed that the polyacrylamide gels were softer than the polystyrene (PS) and polydimethylsiloxane (PDMS) substrates that were used in previous *in vitro* systems.<sup>8</sup> Therefore, the stiffness of the polyacrylamide gels more closely mimics the stiffness of native soft tissue. The cells also behaved differently on the polyacrylamide gels than on PDMS or PS in that a smaller fraction of the cells spread out on the surface of the gel. Further analysis will need to be conducted to determine if this difference is due to differences in substrate materials, stiffness, or surface chemistry.

In the future, migration velocity data will be obtained for MDA-MB-231 cells on 1 kPa and 120 kPa polyacrylamide gels. The stiffness of the gels will also need to be measured using atomic force microscopy (AFM) to assess how the increased initiator concentrations affected the stiffness of the gels. Once the system is verified using the MDA-MB-231 cells, the polyacrylamide gels will be used to obtain migration velocity data for another model of highly migratory cancer, glioblastoma multiforme (GBM). Eventually, FAK activation and F-actin distribution will be quantified in established breast cancer and GBM cell lines, and these factors will be correlated with the migration velocity data for both cell types, providing a more holistic view of cancer cell migration. Relating FAK activation and F-actin distribution to migration rate in established cell lines could also provide a point of comparison for patient-derived cells. Clinicians would be able to compare the FAK activation, F-actin distribution, and migration rate of patient derived cells to those values established for existing breast cancer and GBM cell lines,

which would allow clinicians to more accurately determine prognoses for patients and result in more personalized treatments.

The ultimate goal of this research is to examine cancer cell migration patterns and factors influencing those patterns in an environment that more closely mimics *in vivo* conditions. The biomimicry of the system will allow parallels to be drawn between behaviors of cancer cells *in vitro* to the behavior of the cells *in vivo*, which will result in increasingly personalized care for patients.

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**Appendix A**  
*Supplemental Methodology*

## Polyacrylamide Gel Protocol

1. Prepare a 10% (w/v) solution of ammonium persulfate (APS) in distilled water. (NOTE: APS is highly hygroscopic, do not leave the reagent bottle open for a long period of time. Always close tightly and secure with parafilm. The ammonium persulfate solution must be made fresh daily.)
2. Prepare the gel solutions as follows (per 1000  $\mu\text{L}$ ):

| Desired Modulus (kPa) | % Acrylamide | % Bis-acrylamide | $\mu\text{L}$ from 40% Acrylamide Stock | $\mu\text{L}$ from 2% Bis-Acrylamide Stock | 0.05% TEMED ( $\mu\text{L}$ ) | 0.5% of 10% APS ( $\mu\text{L}$ ) | Distilled Water ( $\mu\text{L}$ ) |
|-----------------------|--------------|------------------|---|--|-------------------------------|-----------------------------------|-----------------------------------|
| 1                     | 5            | 0.03             | 125                                     | 15   | 2                             | 5                                 | 855                               |
| 10                    | 10           | 0.3              | 250                                     | 150  | 2                             | 10                                | 595                               |
| 120                   | 15           | 1.2              | 375                                     | 600  | 2                             | 10                                | 20                                |

3. Mix the gel solution in a vial for 30 seconds.
4. Pipette 100  $\mu\text{L}$  of gel solution onto the SU8 wafer containing the desired micropattern.
5. Place a treated coverslip (treated side down) on the gel solution.
6. Allow 30-45 minutes for the gel to polymerize. (NOTE: The polyacrylamide is fully polymerized when the gel recedes from the edges of the glass coverslip.)
7. Gently remove the gel and coverslip from the SU8 mold with forceps. (NOTE: If the gel cannot be easily removed from the SU8 mold, cover the gel with distilled water and wait 10 minutes before attempting to de-mold the gel again.)
8. Keep the gels covered in distilled water in a Petri dish that is sealed with parafilm.
9. Store the gels at 4°C.



### **Glass Coverslip Treatment Protocol**

1. Rinse the desired number of glass coverslips in 70% ethanol.
2. Soak the coverslips in 0.1N NaOH, then air dry. (NOTE: It is convenient to place the coverslips on wax paper to dry.)
3. Cover the surface of the coverslips with 3-(2-aminoethylamino)propyltrimethoxysilane (or a similar silane) and incubate for 5 minutes inside a fume hood.
4. Wash/immerse the coverslips in distilled water, then air dry.
5. Cover the glass surface with 0.5% glutaraldehyde solution in phosphate buffered saline (PBS) for 30 minutes inside the fume hood.
6. Wash extensively with distilled water, then air dry.

## Image Post-Processing Protocol

1. Download Fiji from <http://fiji.sc/>
2. Open Fiji.
3. Open the .nd2 file that contains the series of images to be analyzed.
4. Select Plugins>Tracking>Manual Tracking.
5. Input the time between images and the size of each pixel in the image (10 minutes and 1.6  $\mu\text{m}$  per pixel for the study described in this thesis).
6. Select add track.
7. Click on the middle of a cell.
8. The manual tracking plugin will step through the images. Keep clicking on the center of the cell as it moves around.
9. Select Overlay Dots & Lines to add a trace to the image
10. Save the Results file as an Excel spreadsheet. The velocity data can be averaged for each cell and for the total number of cells tracked (units of  $\mu\text{m}/\text{min}$  in this case).
11. Save the tracking sequence with the overlay by selecting File>Save As>AVI>JPEG.
12. Repeat the tracking procedure as needed until the desired number of cells have been traced.

For more detailed cell tracking procedures please refer to:

<http://www.kairosinstruments.com/wp-content/uploads/2011/07/Manual-Cell-Tracking-with-Fiji.pdf>

and

<http://rsbweb.nih.gov/ij/plugins/track/Manual%20Tracking%20plugin.pdf>